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A HYBRIDIZATION PROCESS AND A METHOD FOR DETECTING GENETIC VARIATION EMPLOYING SAME AND AN APPARATUS THEREFOR

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(72) Inventor: Jiro [*Illeg.*]
Hitachi, Ltd., Central Research Laboratory
1-280 Higashi-Koigakubo
Kokubunji-shi, Tokyo-to

(72) Inventor: Keiichi Nagai
Hitachi, Ltd., Central Research Laboratories
1-280 Higashi-Koigakubo
Kokubunji-shi, Tokyo-to

(72) Inventor: Daizo Tokinaga
Hitachi, Ltd., Central Research Laboratories
1-280 Higashi-Koigakubo
Kokubunji-shi, Tokyo-to

(71) Applicant: Hitachi, Ltd.
4-6 Kanda Surugadai
Chiyoda-ku, Tokyo-to

(74) Agent: Yusuke Hiraki, Patent Agent, and one other

AQ3

SPECIFICATION

1. Title of Invention

A Hybridization Process and a Method for Detecting Genetic Variation Employing Same and an Apparatus Therefor

2. Claims

1. In the context of a hybridization process for a nucleic acid sample and a nucleic acid probe, a hybridization process for a nucleic acid sample characterized in that a nucleic acid probe is fixed within an electrophoretic carrier and a nucleic acid sample is made to move within the electrical [*probably a typo for* "electrophoretic"] carrier by means of electrophoresis.

2. In the context of a method for detecting genetic variation employing a hybridization reaction of a nucleic acid sample and a nucleic acid probe, a method for detecting genetic variation characterized in that a nucleic acid probe is fixed within an electrophoretic carrier, a nucleic acid sample is made to move within the electrophoretic carrier by means of electrophoresis, a hybridization reaction is caused to be carried out, and [a portion of] the aforesaid nucleic acid sample that has not bonded with the aforesaid nucleic acid probe is made to move by means of electrophoresis and is removed from the aforesaid electrophoretic carrier.

3. In the context of a method for detecting genetic variation employing a hybridization reaction of a nucleic acid sample and a nucleic acid probe, a method for detecting genetic variation characterized in that a nucleic acid probe is fixed within an electrophoretic carrier, a nucleic acid sample is made to move within the electrophoretic carrier by means of electrophoresis, a hybridization reaction is caused to be carried out, following which the aforesaid electrophoretic carrier is then heated and [a portion of] the aforesaid nucleic acid sample that has not bonded with the aforesaid nucleic acid probe is thereafter made to move by means of electrophoresis and is removed from the aforesaid electrophoretic carrier.

4. In the context of a method for detecting genetic variation employing a hybridization reaction of a nucleic acid sample and a nucleic acid probe, a method for detecting genetic variation characterized in that a nucleic acid probe is fixed within an electrophoretic carrier, a nucleic acid sample is made to move within the electrophoretic carrier by means of electrophoresis, a hybridization reaction is caused to be carried out, [a portion of] the aforesaid nucleic acid sample that has not bonded with the aforesaid nucleic acid probe is made to move by means of electrophoresis and is removed from the electrophoretic carrier, a labeled nucleic acid probe is furthermore made to

move within the electrophoretic carrier by means of electrophoresis, a hybridization reaction is caused to be carried out, [a portion of] the aforesaid labeled nucleic acid probe that has not bonded with the aforesaid nucleic acid probe is made to move by means of electrophoresis and is removed from the electrophoretic carrier, and the label of [a portion of] the labeled nucleic acid probe that has bonded with the aforesaid nucleic acid sample is thereafter detected.

5. In the context of a method for detecting genetic variation employing a hybridization reaction of a nucleic acid sample and a nucleic acid probe, a method for detecting genetic variation characterized in that a nucleic acid probe is fixed within an electrophoretic carrier, a nucleic acid sample is made to move within the electrophoretic carrier by means of electrophoresis, a hybridization reaction is caused to be carried out, following which the aforesaid electrophoretic carrier is heated, [a portion of] the aforesaid nucleic acid sample that has not bonded with the aforesaid nucleic acid probe is thereafter made to move by means of electrophoresis and is removed from the aforesaid electrophoretic carrier, a labeled nucleic acid probe is furthermore made to move within the electrophoretic carrier by means of electrophoresis, a hybridization reaction is caused to be carried out, following which the aforesaid electrophoretic carrier is then heated, [a portion of] the aforesaid labeled nucleic acid probe that has not bonded with the aforesaid nucleic acid probe is thereafter made to move by means of electrophoresis and is removed from the electrophoretic carrier, and the label of [a portion of] the labeled nucleic acid probe that has bonded with the aforesaid nucleic acid sample is thereafter detected.

6. A method for detecting genetic variation according to claim 4 or 5 characterized in that the label is a fluorescent substance or pigment, and these are detected within the electrophoretic carrier.

7. A method for detecting genetic variation according to claim 4 or 5 characterized in that the label is an enzyme, and a fluorescent substance or pigment produced as a result of enzymatic reaction caused by said enzyme is either detected within the electrophoretic carrier or is detected [after being] made to move out of the aforesaid electrophoretic carrier by means of electrophoresis.

8. In the context of an apparatus for detecting genetic variation employing a hybridization reaction of a nucleic acid sample and a nucleic acid probe, an apparatus for detecting genetic variation characterized in that it is equipped with an electrophoretic carrier within which is fixed a nucleic acid probe for causing hybridization of a nucleic acid sample, a DC voltage application means that applies, by way of cathode-side electrolytic solution and anode-side electrolytic solution, a DC voltage to the electrophoretic carrier within which

the aforesaid nucleic acid probe is fixed, and a measurement means that measures fluorescence or absorbance of light within the aforesaid electrophoretic carrier or within the aforesaid cathode-side electrolytic solution.

9. An apparatus for detecting genetic variation according to claim 8 characterized in that the measurement means measures fluorescence or absorbance of light within the cathode-side electrolytic solution, and in that provided therein is a membrane, allowing electrolytic solution to pass but not transmitting fluorescent substance or pigment, for concentrating the fluorescent substance or pigment that is within the cathode-side electrolytic solution and that is [to be] measured by the aforesaid measurement means.

10. An apparatus for detecting genetic variation according to claim 8 or 9 characterized in that it is equipped with a means for controlling the temperature of the aforesaid electrophoretic carrier.

11. An electrophoretic carrier within which is fixed a nucleic acid probe that is employed in a hybridization process for a nucleic acid sample and a nucleic acid probe or in a method for detecting genetic variation employing a hybridization reaction of a nucleic acid sample and a nucleic acid probe.

3. Detailed Description of Invention

Industrial Field of Application

The instant invention pertains to a hybridization process for a nucleic acid sample and a method for detecting genetic variation employing same method and an apparatus therefor, and in particular pertains to an apparatus and a method for detecting genetic variation rapidly and in a fashion facilitating automation thereof.

Conventional Art

A conventional method for detecting genetic variation employing a hybridization reaction wherein either a nucleic acid (DNA or RNA) sample or a DNA (RNA) probe (DNA (RNA) fragments possessing base sequences complementary to target DNA (RNA)) is fixed within a solid phase is described at *Proc. Natl. Acad. Sci. USA*, Vol. 80 (1983), pp. 278 - 282.

In this method, a DNA fragment sample separated by molecular weight by means of electrophoresis is first transferred onto a nitrocellulose membrane and fixed thereon, this membrane is thereafter immersed within a solution containing a DNA probe, and a hybridization reaction is carried out. During the hybridization reaction, the higher the degree of complementarity between base sequences therein the stronger will be the bonding between the DNA fragment sample and the DNA probe, dissociation thereof not

occurring even at high temperature. Now, if the DNA fragment sample possesses perfect complementarity with respect to the DNA probe it will not dissociate therefrom, but if there is no complementarity or if there is less than perfect complementarity [the DNA probe can] then be washed [off and removed] at a temperature such as will permit dissociation therefrom. If the DNA fragment sample possesses perfect complementarity with respect to the DNA probe the DNA probe will remain bonded to the membrane and will be left behind, where it can be detected; but if not, the DNA probe will be washed off the membrane and will not be detected. As described above, this method makes it possible to determine whether or not the DNA fragment sample possesses perfect complementarity with respect to the DNA probe. Accordingly, by using a DNA fragment possessing perfect complementarity with respect to a normal target gene as the DNA probe, it is possible to determine whether the target gene within a DNA fragment sample is normal or whether it is abnormal due to the presence of point mutation, insertion, deletion, or other such variation, permitting detection of genetic variation.

Problem to Be Solved by Invention

In the conventional method described above, because the hybridization reaction takes place as a result of passive diffusion between a DNA fragment sample fixed on a nitrocellulose membrane (solid phase) and a DNA probe within solution, there has been the problem that reaction rate is slow. Moreover, there has also been the problem that [the conventional method] comprises operations which do not lend themselves to automation, these being the filling and discharge of the several solutions during carrying out of reaction and during washing.

The object of the instant invention is to provide a hybridization process and a method for detecting genetic variation employing said method and an apparatus for use therein that are rapid, that lend themselves to automation, wherein hybridization reaction rate is fast, and wherein there are few operations that do not lend themselves to automation, such as filling and discharge of solutions and so forth.

Means for Solving Problem

In order to accomplish the aforesaid object, in the instant invention a DNA probe is fixed on an electrophoretic carrier, above and below which are arranged, by way of [intervening] buffer solution, two electrodes, a nucleic acid fragment sample or the like undergoes forced movement by means of electrophoresis, and hybridization reaction(s) and washing are carried out.

That is, the instant invention, in the context of a hybridization process for a nucleic acid sample and a nucleic acid probe, is a hybridization process for a nucleic acid sample characterized in that a nucleic acid probe is fixed within an electrophoretic carrier and a nucleic acid sample is made to move within the electrical [*probably a typo for* "electrophoretic"] carrier by means of electrophoresis. In this hybridization process, because the nucleic acid sample undergoes forced movement across the electrophoretic

carrier on which the DNA probe is fixed, this permits the hybridization reaction to take place more rapidly, and the reaction to be completed in a shorter time, than would be the case with the aforesaid conventional method.

Furthermore, the instant invention, in the context of a method for detecting genetic variation employing a hybridization reaction of a nucleic acid sample and a nucleic acid probe, is a method for detecting genetic variation characterized in that a nucleic acid probe is fixed within an electrophoretic carrier, a nucleic acid sample is made to move within the electrophoretic carrier by means of electrophoresis, a hybridization reaction is caused to be carried out, and [a portion of] the aforesaid nucleic acid sample that has not bonded with the aforesaid nucleic acid probe is made to move by means of electrophoresis and is removed from the aforesaid electrophoretic carrier.

The aforesaid method for detecting genetic variation may be carried out such that it employs two types of nucleic acid probes; i.e., a nucleic acid probe which is fixed on the electrophoretic carrier (the fixed probe), and a labeled second nucleic acid probe (the labeled probe), which is [used to] further hybridize [the portion of] the nucleic acid sample that has bonded to the aforesaid fixed probe. That is, this method for detecting genetic variation may be carried out such that a nucleic acid probe is fixed within an electrophoretic carrier, a nucleic acid sample is made to move within the electrophoretic carrier by means of electrophoresis, a hybridization reaction is caused to be carried out, [a portion of] the aforesaid nucleic acid sample that has not bonded with the aforesaid nucleic acid probe is made to move by means of electrophoresis and is removed from the electrophoretic carrier, a labeled nucleic acid probe is furthermore made to move within the electrophoretic carrier by means of electrophoresis, a hybridization reaction is caused to be carried out, [a portion of] the aforesaid labeled nucleic acid probe that has not bonded with the aforesaid nucleic acid probe is made to move by means of electrophoresis and is removed from the electrophoretic carrier, and the label of [a portion of] the labeled nucleic acid probe that has bonded with the aforesaid nucleic acid sample is thereafter detected.

Moreover, it is possible with any of the aforesaid methods to add an operation wherein the electrophoretic carrier is heated after causing the hybridization reaction to be carried out. It is desirable that the temperature to which [the electrophoretic carrier] is heated be such that dissociation does not occur if the nucleic acid sample possesses perfect complementarity with respect to the nucleic acid probe but such that dissociation will occur if there is no complementarity or if there is less than perfect complementarity. While this temperature will vary depending on the lengths and base sequences of the nucleic acid sample and nucleic acid probe, and depending on the genetic variation being detected; for example, when using a nucleic acid probe that is 19 bases in length to detect a point mutation within the ß-globin gene, a temperature of 55° C is preferred. Also, this heating of the electrophoretic carrier permits an increase in the precision of the method for detecting genetic variation employing a hybridization reaction of a nucleic acid sample and a nucleic acid probe.

As the label substance for the aforesaid labeled nucleic acid probe, one may employ any [suitable substance] so long as it is capable of being detected, and, while ^{32}P or another such radioisotope may be used, it is preferable to employ a fluorescent substance or pigment, or an enzyme that produces a fluorescent substance or pigment as a result of a reaction, and specifically, one may [preferably] employ, for example, fluorescein isothiacynate [*probably a typo for* "isothiocyanate"] (FITC), esterase, or the like. Also, measurement of this fluorescent substance or this pigment may be carried out either within the aforesaid electrophoretic carrier or [after causing the fluorescent substance or pigment] to move out of the aforesaid electrophoretic carrier by means of electrophoresis.

In addition, with respect to an apparatus for detecting genetic variation for the purpose of carrying out the aforesaid method for detecting genetic variation, the instant invention, in the context of an apparatus for detecting genetic variation employing a hybridization reaction of a nucleic acid sample and a nucleic acid probe, is an apparatus for detecting genetic variation characterized in that it is equipped with an electrophoretic carrier within which is fixed a nucleic acid probe for causing hybridization of a nucleic acid sample, a DC voltage application means that applies, by way of cathode-side electrolytic solution and anode-side electrolytic solution, a DC voltage to the electrophoretic carrier within which the aforesaid nucleic acid probe is fixed, and a measurement means that measures fluorescence or absorbance of light within the aforesaid electrophoretic carrier or within the aforesaid cathode-side electrolytic solution. Furthermore, this apparatus for detecting genetic variation may be such that when the measurement means measures fluorescence or absorbance of light within the cathode-side electrolytic solution, there may be provided therein a membrane, allowing electrolytic solution to pass but not transmitting fluorescent substance or pigment, for concentrating the fluorescent substance or pigment that is within the cathode-side electrolytic solution and that is [to be] measured by the aforesaid measurement means. While any [suitable membrane] may be used as this membrane so long as it provides the aforesaid function, one may employ, for example, a porous glass membrane made of quartz.

Furthermore, this apparatus for detecting genetic variation may be equipped with control means for controlling the temperature of the aforesaid electrophoretic carrier.

Moreover, the instant invention concerns an electrophoretic carrier within which is fixed a nucleic acid probe that is employed in the aforesaid hybridization process for a nucleic acid sample and a nucleic acid probe or method for detecting genetic variation employing a hybridization reaction of a nucleic acid sample and a nucleic acid probe.

Action

After adding the DNA fragment sample to the top surface of the electrophoretic carrier, a DC voltage is applied between the two electrodes, and the DNA fragment sample undergoes forced movement within the carrier. This permits the hybridization reaction to take place more rapidly than is the case when the DNA fragment sample is passively diffused.

Furthermore, [the portion of] the DNA fragment sample that did not bond, or that bonded only weakly, during the hybridization reaction is removed by means of electrophoresis. This permits attainment of a method suitable for automation, as washing operations involving filling and discharge of solutions and so forth are [no longer] required.

Moreover, measurement of fluorescence or absorbance of light from the label substance, a hybridization reaction reactant, may be carried out either [while the label is] within the aforesaid electrophoretic carrier or [while it is] within the cathode-side electrolytic solution; furthermore, if measurement is carried out [while the label is] within the latter, the cathode-side electrolytic solution, measurement sensitivity may be increased through the provision of a membrane that concentrates the fluorescent substance or pigment.

Embodiments

Below, we describe the instant invention in further detail through the use embodiments; however, the instant invention is not to be limited by these embodiments.

EMBODIMENT 1

We describe the instant embodiment with reference to Fig. 1 (a) and (b).

An electrophoretic carrier 1 within which a DNA probe [was] fixed [was] first prepared as follows. The DNA probe [was prepared] by using the phosphoamidide method, currently in wide use, to synthesize a DNA fragment (3'-GAGGACTCCTCTTCAGACG-5') that was perfectly complementary to the base sequence from the 14th to the 32nd [base] from the 5' end of the human ß-globin gene. However, at the final step of synthesis, i.e. the step of adding guanine (G) at the 5' end, we used the method of L.M. Smith et al, wherein deoxyguanosine containing an amino group at its 5' end is employed instead of deoxyguanosine, to introduce an amino group at the 5' end of the DNA fragment. After purifying this DNA probe using high-performance liquid chromatography (HPLC), we then added [the purified DNA probe] to a 2.5% aqueous solution of acrolein and allowed this to react for 30 min over an ice bath. After dialyzing this well using PBS buffer solution, we further added 5% [of] acrylamide - N,N'-methylenebisacrylamide solution (acrylamide : N,N'-methylenebisacrylamide = 20 : 1), N,N,N',N'-tetramethylethylene diamine for a final concentration of 0.08%, and ammonium persulfate for a final concentration of 0.1%, and poured this into a glass tube 2 and allowed this to gel to obtain an electrophoretic carrier 1.

As the DNA fragment sample, we used normal, unmutated human ß-globin gene ($ß^A$) and we used ß-globin gene ($ß^B$) from a patient suffering from sickle cell anemia, wherein the adenosine [*may be a typo for* "adenine"] (A) at the 20th [base] from the 5' end had mutated (point mutation) to thymine (T), which had been broken [into fragments]

using restriction enzyme BamHI (fragments approximately 1,800 base pairs in length including region in vicinity of 5' end of ß-globin gene).

After using heat to denature the aforesaid DNA fragment sample, forming single-stranded DNA, this was poured onto the top end of the electrophoretic carrier 1, on which the DNA probe had been fixed and which was being maintained at 45° C by means of a temperature controller 3, and a DC power supply 10 was used to apply a voltage between an anode 6, present within an upper electrolytic solution tank 4, and a cathode 9, present within a lower electrolytic solution tank 7. Because this causes the DNA fragment sample to undergo forced [movement] into the electrophoretic carrier 1 by means of electrophoresis, the hybridization reaction can proceed more rapidly than would be the case with no electrophoresis, when [the DNA fragment sample] is passively diffused.

Then, after using the temperature controller 3 to change the temperature of the electrophoretic carrier 1 to 55° C, a voltage was again applied between the two electrodes 6,9, and the [portion of the] DNA fragment sample that was dissociated because of lack of perfect complementarity with respect to the DNA probe was removed by means of electrophoresis.

Furthermore, after returning the temperature of the electrophoretic carrier to 45° C, a second DNA probe, which had been labeled with esterase, was poured onto the top end of the electrophoretic carrier 1, and electrophoresis was carried out. This DNA probe (the labeled probe) was a DNA fragment (3'-CCACTTGCACCTACTTCAAC-5') synthesized using the phosphoamidide method in the same manner as the probe fixed on the electrophoretic carrier 1 (the fixed probe), the 5' end thereof being labeled with esterase, but complementary with respect to a different region of the ß-globin gene than the fixed probe; to wit, to the base sequence from the 53rd to the 72nd [base] from the 5' end thereof. Accordingly, if the DNA fragment sample bonds to the fixed probe and remains within the electrophoretic carrier 1, the labeled probe will bond to a different region of the DNA fragment sample and will likewise remain within the electrophoretic carrier 1; however, if the DNA fragment sample does not remain [within the electrophoretic carrier 1], the labeled probe will not remain within the electrophoretic carrier 1 but will pass therethrough.

Finally, FDA (fluorescein diacetate), which acts as substrate for the labeled esterase enzyme, was likewise poured onto the top end of the electrophoretic carrier 1, electrophoresis was carried out, and fluorescence of fluorescein, the fluorescent substance produced by the enzymatic reaction, was thereafter measured within the electrophoretic carrier 1.

Light exiting from a xenon lamp light source 11 was made to pass through an interference filter 12, light of wavelength 490 nm being selected, following which this was condensed by a lens 13 and the electrophoretic carrier 1 was irradiated with excitation light. From a direction that was 90° with respect to the excitation light, [after] passing through a lens 17, a cutoff filter 18, and an interference filter 19, light of wavelength in the

vicinity of 510 nm was selectively detected at a photomultiplier 20. Moreover, a window 16 was provided at the side opposite an incident[-side] window 14, and the effect of scattered light was reduced by guiding to the outside [some of the] excitation light that had passed through the electrophoretic carrier 1. The output from the photomultiplier 20 was amplified at an amplifier 21, and this was thereafter recorded on a recorder 22.

As a result of measurement, [it was found that] with the DNA fragment sample containing normal, unmutated human ß-globin gene ($ß^A$) fragments and for which there was perfect complementarity with respect to the fixed DNA probe, fluorescence was detected; but with the DNA fragment sample containing ß-globin gene ($ß^B$) fragments from a patient suffering from sickle cell anemia, in which there was a mutation (point mutation) and for which complementarity with respect to the fixed DNA probe was lacking only at a single base, fluorescence was not detected. In order to confirm [this result], we replaced the fixed DNA probe with a [fragment] possessing perfect complementarity (3'-GAGGACACCTCTTCAGACG-5') with respect to the $ß^B$ gene and carried out measurements in the same fashion [as before], upon which [it was found that] fluorescence was not detected for the DNA fragment sample containing $ß^A$ gene fragments, but [fluorescence] was detected for the [DNA fragment sample containing] $ß^B$ gene fragments. Because it was possible to distinguish between gene fragments containing variation and gene fragments not containing variation based on whether or not fluorescence was detected, we were thus able to detect variation (point mutation) present within the ß-globin gene fragments.

Moreover, whereas in the instant embodiment we employed an enzyme (esterase) as the label substance and measured fluorescence of FDA produced as a result of enzymatic reaction, one may also employ FITC or other such fluorescent substance as label substance and measure the fluorescence thereof directly without employment of an enzyme or enzymatic reaction.

As described above, the instant embodiment permits attainment of an apparatus and method for detecting genetic variation rapidly and in a fashion facilitating automation thereof.

EMBODIMENT 2

Next, we describe a second embodiment with reference to Fig. 2.

The difference between the instant embodiment and Embodiment 1 is that fluorescence of the fluorescein fluorescent substance [was] measured not within the electrophoretic carrier 1 but within the lower electrolytic solution 8. After causing the FDA to move into the electrophoretic carrier 1 by means of electrophoresis at the last step of the above embodiment, electrophoresis was again continued, causing the fluorescein fluorescent substance produced as a result of enzymatic reaction to migrate into the lower electrolytic solution 8. In addition, fluorescence of fluorescein within the lower electrolytic solution was measured using the apparatus shown at Fig. 2.

In addition to benefits similar to those of the above embodiment, because fluorescence of fluorescein is measured not within the electrophoretic carrier, which displays much scattering of light and interfering fluorescence, but within the electrolytic solution, which displays little of these, the instant embodiment possesses the benefit that it allows fluorescence to be measured with high sensitivity.

EMBODIMENT 3

Next, we describe a third embodiment with reference to Fig. 3.

The difference between the instant embodiment and Embodiment 2 is the fact that a small-volume electrolytic solution tank 25 is constituted as a result of arrangement of a porous glass membrane 24 attached to a membrane retaining fixture 23 made of acrylic between the bottom end of the electrophoretic carrier 1 and the lower electrolytic solution 8. The aforesaid porous glass membrane 24 is quartz glass that, having been reacted with tetramethoxysilane in a solvent containing methanol and water according to the sol-gel method, possesses properties such that it allows the electrolyte(s) of the electrolytic solution to be transmitted [i.e., to pass] but does not allow the fluorescent substance to be transmitted [i.e., to pass]. Accordingly, FDA fluorescent substance produced as a result of enzymatic reaction will be concentrated within the small-volume electrolytic solution tank 25. In the instant embodiment, a pipette 27 was used to guide electrolytic solution containing fluorescent substance concentrated as a result of the above process through a guide hole 26 and into a fluorescence cell 28. The pipette 27 was retained by a mechanism 29 [capable of] rotary and vertical [movement]. Fluorescence of the fluorescent substance within the fluorescence cell 28 was measured using an optical system similar to that shown in Fig. 2.

In addition to benefits similar to those of Embodiment 2, because the instant invention permits the FDA fluorescent substance to be concentrated within a small volume of electrolytic solution, it possesses the benefit that it allows fluorescence to be measured with even higher sensitivity.

Benefit of Invention

In the instant invention, because the DNA fragment sample undergoes forced movement within the electrophoretic carrier by means of electrophoresis, this permits the hybridization reaction to take place more rapidly, and the reaction to be completed in a shorter time, than would be the case were it to undergo passive diffusion as in the conventional method employing a nitrocellulose membrane. Furthermore, [the instant invention permits] easy removal, by means of electrophoresis, without employment of washing operations involving filling and discharge of solutions and so forth, of [the portion of] the DNA sample that does not bond, or that bonds only weakly, during the hybridization reaction. Accordingly, [the instant invention] permits attainment of a method for detecting genetic variation rapidly and in a fashion facilitating automation thereof.

Moreover, the instant invention permits an increase in measurement sensitivity as a result of concentration of the fluorescent substance or pigment [used as] label substance.

4. Brief Description of Drawings

Fig. 1 (a) and (b) are, respectively, a longitudinal cross-section and a lateral cross-section of an apparatus used in a first embodiment of the instant invention, Fig. 2 is a longitudinal cross-section of an apparatus used in a second embodiment of the instant invention, and Fig. 3 is an enlarged view of a portion of a longitudinal cross-section of an apparatus used in a third embodiment of the instant invention.

1...electrophoretic carrier; 2...glass tube; 3...temperature controller; 4...upper electrolytic solution tank; 5...upper (anode-side) electrolytic solution; 6...anode; 7...lower (cathode-side) electrolytic solution tank; 8...lower (cathode-side) electrolytic solution; 9...cathode; 10...DC power supply; 11...light source; 12,19...interference filter; 13,17...lens; 14...incident[-side] window; 15...detection window; 16...window; 18...cutoff filter; 20...photomultiplier; 21...amplifier; 22...recorder; 23...membrane retaining fixture; 24...porous glass membrane; 25...small-volume electrolytic solution tank; 26...guide hole; 27...pipette; 28...fluorescence cell; 29...mechanism [capable of] rotary and vertical [movement].

Fig. 1

Fig. 2

Fig. 3

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⑭発明の名称　ハイブリダイゼーション方法、これを用いた遺伝子変異検出方法及びその装置

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⑰発明者　鴇田　二郎　東京都国分寺市東恋ケ窪1丁目280番地　株式会社日立製作所中央研究所内
⑰発明者　永井　啓一　東京都国分寺市東恋ケ窪1丁目280番地　株式会社日立製作所中央研究所内
⑰発明者　時永　大三　東京都国分寺市東恋ケ窪1丁目280番地　株式会社日立製作所中央研究所内
⑪出願人　株式会社日立製作所　東京都千代田区神田駿河台4丁目6番地
⑭代理人　弁理士 平木 祐輔　外1名

明細書

1．発明の名称

ハイブリダイゼーション方法、これを用いた遺伝子変異検出方法及びその装置

2．特許請求の範囲

1．核酸プローブと核酸試料のハイブリダイゼーション方法において、核酸プローブを電気泳動担体中に固定し、核酸試料を電気泳動によって電気担体中に移動せしめることを特徴とする核酸試料のハイブリダイゼーション方法。

2．核酸プローブと核酸試料のハイブリダイゼーション反応を用いた遺伝子変異検出法において、核酸プローブを電気泳動担体中に固定し、核酸試料を電気泳動によって電気泳動担体中に移動せしめてハイブリダイゼーション反応を行なわせ、上記核酸プローブと結合しなかった上記核酸試料を電気泳動によって移動せしめて上記電気泳動担体中から除去することを特徴とする遺伝子変異検出方法。

3．核酸プローブと核酸試料のハイブリダイゼーション反応を用いた遺伝子変異検出法において、核酸プローブを電気泳動担体中に固定し、核酸試料を電気泳動によって電気泳動担体中に移動せしめてハイブリダイゼーション反応を行わせ、次いで前記電気泳動担体を加温した後、上記核酸プローブと結合しなかった上記核酸試料を電気泳動によって移動せしめて上記電気泳動担体中から除去することを特徴とする遺伝子変異検出方法。

4．核酸プローブと核酸試料のハイブリダイゼーション反応を用いた遺伝子変異検出法において、核酸プローブを電気泳動担体中に固定し、核酸試料を電気泳動によって電気泳動担体中に移動せしめてハイブリダイゼーション反応を行なわせ、上記核酸プローブと結合しなかった上記核酸試料を電気泳動によって移動せしめて電気泳動担体中から除去し、さらに標識核酸プローブを電気泳動によって電気泳動担体中に移動せしめてハイブリダイゼーション反応を行なわせ、上記核酸プローブと結合しなかった上記標識核

酸プローブを電気泳動によって移動せしめて電気泳動担体中から除去した後、上記核酸試料と結合した標識核酸プローブの標識を検出することを特徴とする遺伝子変異検出方法。

5．核酸プローブと核酸試料のハイブリダイゼーション反応を用いた遺伝子変異検出法において、核酸プローブを電気泳動担体中に固定し、核酸試料を電気泳動によって電気泳動担体中に移動せしめてハイブリダイゼーション反応を行わせ、次いで前記電気泳動担体を加温した後、上記核酸プローブと結合しなかった上記核酸試料を電気泳動によって移動せしめて上記電気泳動担体中から除去し、さらに標識核酸プローブを電気泳動によって電気泳動担体中に移動せしめてハイブリダイゼーション反応を行わせ、次いで上記電気泳動担体を加温した後、上記核酸プローブと結合しなかった上記標識核酸プローブを電気泳動によって移動せしめて電気泳動担体中から除去した後、上記核酸試料と結合した標識核酸プローブの標識を検出することを特徴とする遺伝子変異検出方法。

6．標識は蛍光体又は色素であり、これらを電気泳動担体中で検出することを特徴とする請求項4又は5記載の遺伝子変異検出方法。

7．標識は酵素であり、当該酵素による酵素反応によって生成する蛍光体又は色素を電気泳動担体中で検出するか、あるいは電気泳動によって上記電気泳動担体中から外に移動せしめて検出することを特徴とする請求項4又は5記載の遺伝子変異検出方法。

8．核酸プローブと核酸試料のハイブリダイゼーション反応を用いた遺伝子変異検出装置において、核酸試料をハイブリダイゼーションさせるための核酸プローブを固定した電気泳動担体と、上記核酸プローブを固定した電気泳動担体に正極側電解液と負極側電解液を介して直流電圧を印加する直流電圧印加手段と、上記電気泳動担体中又は上記正極側電解液中の蛍光又は光の吸収を計測する計測手段とを具備したことを特徴とする遺伝子変異検出装置。

9．計測手段が正極側電解液中の蛍光又は光の吸収を計測する手段であって、前記計測手段によって計測される正極側電解液中の蛍光体又は色素を濃縮するための、電解液は通過するが蛍光体又は色素は通過しない膜を設けたことを特徴とする請求項8記載の遺伝子変異検出装置。

10．上記電気泳動担体の温度をコントロールする手段を具備したことを特徴とする請求項8又は9記載の遺伝子変異検出装置。

11．核酸プローブと核酸試料のハイブリダイゼーション方法又は核酸プローブと核酸試料のハイブリダイゼーション反応を用いた遺伝子変異検出法に用いる核酸プローブを固定した電気泳動担体。

3．発明の詳細な説明

（産業上の利用分野）

本発明は核酸試料のハイブリダイゼーション方法、この方法を用いた遺伝子変異検出方法及びその装置に関し、特に高速で自動化容易な遺伝子変異検出方法及び装置に関する。

（従来の技術）

核酸（ＤＮＡ又はＲＮＡ）試料又はＤＮＡ（ＲＮＡ）プローブ（標的ＤＮＡ（ＲＮＡ）と相補的な塩基配列を持つＤＮＡ（ＲＮＡ）断片）のいずれか一方を固相に固定したハイブリダイゼーション反応を用いる従来の遺伝子変異検出法は、プロシーディングス　オブ　ナチュラルアカデミー　オブ　サイエンス　ユー　エス　エー，80巻（1983年）第278頁から282頁（Proc.Natl. Acad.Sci. USA, 80, (1983), pp.278 ～282)に記載されている。

この方法は、まず、電気泳動によって分子量分離したＤＮＡ断片試料をニトロセルロース膜上に転写、固定した後、この膜をＤＮＡプローブを含む溶液に浸してハイブリダイゼーション反応を行なう。ハイブリダイゼーション反応では、塩基配列の相補性が高い程、ＤＮＡ断片試料とＤＮＡプローブは強く結合し、高い温度でも解離することがない。そこで、次に、ＤＮＡ断片試料がＤＮＡプローブと完全な相補性をもつ場合には解離せず、

相補性がないか又は相補性が不完全な場合には解離するような温度で洗浄を行なう。ＤＮＡ断片試料がＤＮＡプローブと完全な相補性を持つものである場合には、ＤＮＡプローブは膜に結合したまま残って検出されるが、そうでない場合には、ＤＮＡプローブは膜から洗い流されて検出されない。以上のように、この方法では、ＤＮＡ断片試料がＤＮＡプローブと完全な相補性を持つか否かを判定できる。したがって、正常な標的遺伝子と完全な相補性を持つＤＮＡ断片をＤＮＡプローブとすることにより、ＤＮＡ断片試料中の標的遺伝子が正常なものか、あるいはポイントミューテーション、挿入、欠失等の変異を含む異常なものかを判定でき、遺伝子の変異を検出できる。

〔発明が解決しようとする課題〕

上記の従来法では、ハイブリダイゼーション反応がニトロセルロース膜（固相）に固定されたＤＮＡ断片試料と、溶液中のＤＮＡプローブの受動的拡散によって起こるため、反応速度が遅いという問題点があった。また、反応時及び洗浄時には、各々の溶液の注入、排出という自動化しにくい動作が含まれているという問題点があった。

本発明の目的は、ハイブリダイゼーションの反応速度が速く、しかも溶液の注入、排出等の自動化しにくい動作の少ない、高速で自動化容易なハイブリダイゼーション方法、該方法を用いた遺伝子変異検出法及びそれに用いる装置を提供することにある。

〔課題を解決するための手段〕

上記目的を達成するために、本発明では、ＤＮＡプローブを電気泳動担体に固定し、その上下に膜溶液を介して２つの電極を配置して、電気泳動により核酸断片試料等を強制的に移動させて、ハイブリダイゼーション反応や洗浄を行なうようにした。

即ち、本発明は、核酸プローブと核酸試料のハイブリダイゼーション方法において、核酸プローブを電気泳動担体中に固定し、核酸試料を電気泳動によって電気担体中に移動せしめることを特徴とする核酸試料のハイブリダイゼーション方法である。このハイブリダイゼーション方法によれば、ＤＮＡプローブを固定した電気泳動担体上を核酸試料を強制的に移動させるものであるから、ハイブリダイゼーション反応が、上記従来法に比して速く、この反応を短時間で完了することができる。

さらに本発明は、核酸プローブと核酸試料のハイブリダイゼーション反応を用いた遺伝子変異検出法において、核酸プローブを電気泳動担体中に固定し、核酸試料を電気泳動によって電気泳動担体中に移動せしめてハイブリダイゼーション反応を行わせ、上記核酸プローブと結合しなかった上記核酸試料を電気泳動によって移動せしめ上記電気泳動担体中から除去することを特徴とする遺伝子変異検出方法である。

上記遺伝子変異検出法においては、２種類の核酸プローブ、即ち、電気泳動担体に固定する核酸プローブ（固定化プローブ）と、前記固定化プローブに結合した核酸試料に更にハイブリダイズする標識化された第２の核酸プローブ（標識プローブ）を用いて行うことができる。即ち、この遺伝子変異検出法は、核酸プローブを電気泳動担体中に固定し、核酸試料を電気泳動によって電気泳動担体中に移動せしめてハイブリダイゼーション反応を行わせ、上記核酸プローブと結合しなかった上記核酸試料を電気泳動によって移動せしめて電気泳動担体中から除去し、さらに標識核酸プローブを電気泳動によって電気泳動担体中に移動せしめてハイブリダイゼーション反応を行わせ、上記核酸プローブと結合しなかった上記標識核酸プローブを電気泳動によって移動せしめて電気泳動担体中から除去した後、上記核酸試料と結合した標識核酸プローブの標識を検出することにより行うことができる。

また、上記いずれの方法においてもハイブリダイゼーション反応を行わせた後、電気泳動担体を加温する工程を加えることができる。加温する温度は、核酸試料が核酸プローブと完全な相補性をもつ場合には解離せず、相補性がないか又は相補性が不完全な場合には解離するような温度が好ましい。この温度は、核酸試料と核酸プローブの長

さと塩基配列及び検出しようとする遺伝子の変異によって種々異なるが、例えば、β-グロビン遺伝子中のポイントミューテーションを19塩基長の核酸プローブで検出する場合は55℃が好ましい。そして、この電気泳動担体の加温により、核酸プローブと核酸試料とのハイブリダイゼーション反応を用いた遺伝子変異検出法の精度を高めることができる。

上記標識核酸プローブの標識物質としては、検出可能なものであればいずれでもよく、^{32}P等のラジオアイソトープでもよいが、好ましくは蛍光体又は色素若いは反応により蛍光体又は色素を生成する酵素が用いられ、具体的には例えばフルオレセイン イソチアシネート(FITC)、エステラーゼ等が用いられる。そして、これらの蛍光体又は色素の計測は、上記電気泳動担体中あるいは電気泳動により上記電気泳動担体中から外に移動せしめたものについてのいずれにおいても行うことができる。

さらに、本発明は、上記遺伝子変異検出方法を実施するための遺伝子変異検出装置に係わり、核酸プローブと核酸試料のハイブリダイゼーション反応を用いた遺伝子変異検出装置において、核酸試料をハイブリダイゼーションさせるための核酸プローブを固定した電気泳動担体と、上記核酸プローブを固定した電気泳動担体に正極側電解液と負極側電解液を介して直流電圧を印加する直流電圧印加手段と、上記電気泳動担体中又は上記正極側電解液中の蛍光又は光の吸収を計測する計測手段とを具備したことを特徴とする遺伝子変異検出装置である。また、この遺伝子変異検出装置は、計測手段が、正極側電解液中の蛍光又は光の吸収を計測する手段である場合は、前記計測手段によって計測される正極側電解液中の蛍光体又は色素を濃縮するための、電解液は通過するが蛍光体又は色素は透過しない膜を設けることができる。この膜は上記緩衝を備えるものであればいずれでもよいが、例えば石英製のポーラスガラス膜が用いられる。

また、この遺伝子変異検出装置には上記電気泳動担体の温度をコントロールするためのコントロール手段を備えることができる。

さらに本発明は、上記核酸プローブと核酸試料のハイブリダイゼーション方法又は核酸プローブと核酸試料のハイブリダイゼーション反応を用いた遺伝子変異検出法に用いる核酸プローブを固定した電気泳動担体に係るものである。

〔作　用〕

電気泳動担体の上面にＤＮＡ断片試料を添加した後、２つの電極間に直流電圧を印加して、ＤＮＡ断片試料を強制的に担体中に移動させる。これにより、ＤＮＡ断片試料を受動的に拡散させる場合よりも、ハイブリダイゼーション反応を速くできる。

また、ハイブリダイゼーション反応で結合しなかったか又は結合が弱かったＤＮＡ断片試料を電気泳動により除去する。これにより、溶液の注入、排出等による洗浄操作が不要な、自動化に適した方法を実現できる。

さらに、ハイブリダイゼーション反応物の標識物質からの蛍光又は光の吸収の計測は上記電気泳動担体あるいは正極側の電解液中のいずれにおいても行うことができ、また、後者の正極側の電解液中で計測する場合は、蛍光体又は色素を濃縮する膜を備えることにより計測の感度が高められる。

〔実施例〕

以下、本発明を実施例により詳細に説明する。但し、本発明はこれらの実施例により限定されるものでない。

実施例１

本実施例を第１図(a)、(b)により説明する。

まず、ＤＮＡプローブを固定した電気泳動担体１は以下のようにして調製する。ＤＮＡプローブは、ヒトβ-グロビン遺伝子の5'末端から14～32番目の塩基配列と完全に相補的なＤＮＡ断片（5'-GAGGAGTCCTCTTCAGACG-3'）を、現在広く用いられているフォスフォアミダイド法で合成した。ただし、合成の最終ステップ、すなわち5'末端のグアニン(G)を付加するステップでは、デオキシグアノシンのかわりに5'末端にアミノ基をもつデオキ

シグアノシンを用いるL.M.Smith らの方法により、DNA断片の5'末端にアミノ基を導入した。次に、このDNAプローブを高速液体クロマトグラフィー(HPLC)で精製した後、2.5%アクロレイン水溶液に加えて氷冷下30分間反応させた。これをPBS緩衝液でよく透析した後、さらに5%アクリルアミド-N, N'-メチレンビスアクリルアミド溶液(アクリルアミド:N, N'-メチレンビスアクリルアミド=20:1)、最終濃度0.08%のN, N, N', N'-テトラメチルエチレンジアミン、最終濃度0.1%の過硫酸アンモニウムを加えてガラス管2に注入し、ゲル化させて電気泳動担体1とした。

DNA断片試料としては、変異を含まない正常人のβ-グロビン遺伝子（β^A）と5'末端から20番目のアデノシン(A)がチミン(T)に変異した（ポイントミューテーション）、鎌状赤血球貧血症患者のβ-グロビン遺伝子（β^S）を制限酵素 BamHI で切断したもの（β-グロビン遺伝子の5'末端付近を含む、長さ約1800塩基対の断片）を使用した。

上記DNA断片試料を加熱変性させて一本鎖DNAとしてから、温度コントローラ3によって45℃に保たれているDNAプローブを固定した電気泳動担体1の上端に注入し、上部電解液槽4中の負極6と下部電解液槽7中の正極9の間に直流電源10で電圧を印加した。これにより、DNA断片試料は電気泳動担体1の中へ強制的に電気泳動されるため、電気泳動を行なわずに受動的に拡散させる場合に比べて、ハイブリダイゼーション反応を速く進めることができる。

次に、電気泳動担体1の温度を温度コントローラ3によって55℃に変更してから、再び2つの電極6，9の間に電圧を印加し、DNAプローブと完全な相補性を持たないために解離したDNA断片試料を電気泳動により除去した。

さらに、電気泳動担体の温度を45℃にもどしてから、エステラーゼで標識した第二のDNAプローブを電気泳動担体1の上端に注入し、電気泳動した。このDNAプローブ（標識プローブ）は、電気泳動担体1に固定したプローブ（固定化プローブ）と同様にフォスフォアミダイド法で合成したDNA断片（3'-CCACTTGCACCTACTTCAAC-5'）の5'末端をエステラーゼで標識したものであるが、固定化プローブとは異なる部位、すなわちβ-グロビン遺伝子の5'末端から53～72番目の塩基配列に相補的である。したがって、DNA断片試料が固定化プローブに結合して電気泳動担体1中に残っていれば、標識プローブもDNA断片試料の別の部位に結合して電気泳動担体1中に残るが、DNA断片試料が残っていなければ、標識プローブは電気泳動担体1中に残らず通過する。

最後に、標識酵素エステラーゼの基質であるFDA(フルオレセインジアセテート)を同様に電気泳動担体1の上端に注入して電気泳動した後、酵素反応で生じた蛍光物質フルオレセインの蛍光を、電気泳動担体1中で測定した。

キセノンランプの光源11から出た光を干渉フィルター12に通して490nm の波長の光を選択した後、レンズ13で集光して電気泳動担体1に励起光を照射した。励起光に対して90° の方向から、レンズ17、カットオフフィルター18、干渉フィルター19を通して、510nm 近傍の波長の光を選択的にフォトマル20で検出した。なお、入射窓14の反対側に窓16を設け、電気泳動担体1を通過した励起光を外部に導くことにより、散乱光の影響を少なくした。フォトマル20の出力は増幅器21で増幅した後、レコーダ22で記録した。

測定の結果、DNA断片試料が変異を含まない正常人のβ-グロビン遺伝子（β^A）の断片で、固定化DNAプローブと完全な相補性をもつ場合には、蛍光が検出されたが、DNA断片試料が変異（ポイントミューテーション）を含む鎌状赤血球貧血症患者のβグロビン遺伝子(β^S)の断片で、固定化DNAプローブと1塩基だけ相補性をもたない場合には、蛍光は検出されなかった。確認のために、固定化DNAプローブをβ^S 遺伝子に完全な相補性をもつもの（5'-GAGGACACCTCTTCAGACG-5'）にかえて同様の測定を行なったところ、DNA断片試料がβ^A 遺伝子の断片の場合には蛍光が検出されず、β^S 遺伝子の断片の場合には蛍光が検出された。このように、変異を含む遺伝子断片

と含まない遺伝子断片を、蛍光が検出されるか否かによって区別できるため、β-グロビン遺伝子断片中の変異（ポイントミューテーション）を検出することができた。

なお、本実施例では標識物質として酵素（エステラーゼ）を用い、酵素反応によって生成するFDAの蛍光を測定したが、標識物質としてFITC等の蛍光物質を用い、酵素や酵素反応を用いずに、直接その蛍光を測定してもよい。

以上のように、本実施例により、高速で自動化容易な遺伝子変異検出法及び装置を実現できた。

実施例2

次に、第2の実施例を第2図により説明する。本実施例と実施例1の違いは、蛍光物質フルオレセインの蛍光を、電気泳動担体1中ではなく、下部電解液8中で測定するところにある。前記実施例の最後のステップで、FDAを電気泳動により電気泳動担体1中に移動させた後、さらに電気泳動を続けて酵素反応で生じた蛍光物質フルオレセインを下部電解液8中に泳動させた。そして、フルオレセインの蛍光を第2図に示す装置を用いて、下部電解液中で測定した。

本実施例によれば、前記実施例と同様の効果に加えて、散乱光と励起蛍光の大きい電気泳動担体中ではなく、これらの小さい電解液中でフルオレセインの蛍光を測定するので、高感度な蛍光測定が可能であるという効果がある。

実施例3

次に、第3の実施例を第3図により説明する。本実施例と実施例2の違いは、電気泳動担体1の下端と下部電解液8の間にアクリル製の膜保持具23に取り付けたポーラスガラス膜24を配置することにより、小容積の電解液槽25を構成した点にある。上記ポーラスガラス膜24は、ゾルゲル法でテトラメトキシシランをメタノールと水溶媒中で反応させたもので、電解液中の電解質は透過させるが、蛍光体は透過させないという性質をもつ石英ガラスである。したがって、酵素反応によって生成した蛍光体FDAは小容積の電解液槽25中に濃縮される。本実施例では、上記過程によって濃縮された蛍光体を含む電解液をガイド穴26を通してピペット27を用いて蛍光セル28に導いた。ピペット27は回転上下機構29に保持した。蛍光セル28中の蛍光体は、第2図に示したのと同様な光学系で蛍光計測した。

本実施例によれば、実施例2と同様の効果に加えて、蛍光物質FDAを小容積の電解液中に濃縮できるため、さらに高感度な蛍光測定が可能であるという効果がある。

〔発明の効果〕

本発明によれば、DNA断片試料を電気泳動により強制的に電気泳動担体中に移動させるので、従来のニトロセルロース膜で用いた方法で受動的に拡散させる場合よりも、ハイブリダイゼーション反応を速くでき、短時間で完了できる。また、ハイブリダイゼーション反応で結合しなかったか又は結合が弱かったDNA試料を、溶液の注入、排出等による洗浄操作を用いずに、電気泳動によって容易に除去することができる。したがって、本発明によれば高速で自動化容易な遺伝子変異検出方法を実現できる。更に本発明は、標識物質の蛍光体又は色素を濃縮することにより計測感度を高めることができる。

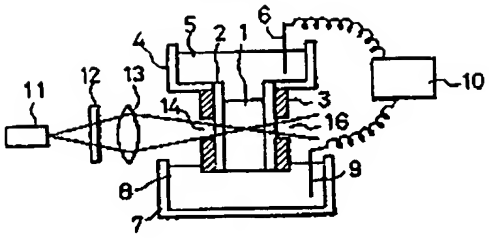
4．図面の簡単な説明

第1図(a)，(b)は各々本発明の第一の実施例で用いた装置の縦断面図と横断面図、第2図は本発明の第二の実施例で用いた装置の縦断面図、第3図は本発明の第三の実施例で用いた装置の縦断面図の一部拡大図である。

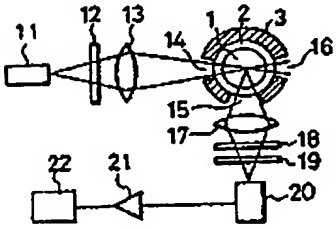
1…電気泳動担体、2…ガラス管、3…温度コントローラ、4…上部電解液槽、5…上部（負極側）電解液、6…負極、7…下部（正極側）電解液槽、8…下部（正極側）電解液、9…正極、10…直流電源、11…光源、12，19…干渉フィルター、13，17…レンズ、14…入射窓、15…検出窓、16…窓、18…カットオフフィルター、20…フォトマル、21…増幅器、22…レコーダー、23…膜保持具、24…ポーラスガラス膜、25…小容積の電解液槽、26…ガイド穴、27…ピペット、28…蛍光セル、29…回転上下機構。

第 1 図

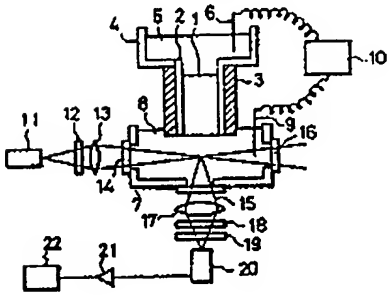
(a)



(b)



第 2 図



第 3 図